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Document Contact	Sharon Scalise: Supv, Laboratory
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Histology Muscle Enzyme - Combined Cytochrome Oxidase - SDH Stain - Royal Oak

Document Type: Procedure

I. PURPOSE AND OBJECTIVE:

The purpose of this document is to provide a procedure for the demonstration of a type of oxidoreductase enzyme known as cytochrome oxidase. This oxidative enzyme catalyzes the reaction between the substrate and oxygen. It is found in the mitochondria. Absence of this enzyme is associated with a progressive muscle weakness that is usually fatal in childhood. This stain can be used for muscle typing, Type I stains darker the Type II, as it has more mitochondria. This stain can also be used to indicate architectural changes in the muscle, such as swirls, target cells, and central core disease, all of which have a displacement of the mitochondria. It will also show depletion or clumping of the mitochondria. Additionally, this purpose is to provide a procedure for the demonstration of succinic dehydrogenase. Succinic dehydrogenase is an anaerobic oxidative enzyme that removes a hydrogen ion from a substrate. It is found in the mitochondria and is involved in the Krebs cycle. This stain can be used for muscle typing, as Type I stains darker than Type II, as it has more mitochondria. This stain can also be used to indicate architectural changes in the muscle, such as swirls, target cells, and central core disease, all of which have a displacement of the mitochondria. Red-ragged fibers, which contain an accumulation of bizarre mitochondria around the rim of each fiber, are especially evident with this stain. Denervated muscles stain dark.

II. PRINCIPLE:

A. The succinic dehydrogenase reaction is an oxidation-reduction reaction. Sodium succinate is the substrate. The succinic dehydrogenase enzymes in the muscle will remove a hydrogen from the sodium succinate (=oxidation). This hydrogen then reduces the tetrazolium salt, nitro-blue tetrazolium (NBT). This forms a highly colored formazan dye which is finely granular blue.

Sodium phosphate and potassium phosphate are used as buffers.

Sodium succinate + Succinic dehydrogenase (tissue) →fumarate + H^+

H^++Nitro blue tetrazolium (NBT) → reduced tetrazolium → formazan (blue)

B. The cytochrome oxidase reaction is an oxidation-reduction reaction. Hydrogen peroxide is the substrate. The cytochrome oxidase enzymes in the muscle will split the hydrogen peroxide into water and oxygen. The oxygen will oxidize the dye, which is 3,3'-diaminobenzidine (DAB). This forms a brown precipitate at the sites of enzyme activity. Sodium acetate is the buffer. Manganese chloride is an activator. This reaction will only happen in the presence of manganese.

Hydrogen peroxide (H_2O_2) + cytochrome oxidase (tissue) → H_2O + O_2

O_2 + DAB → oxidized DAB → brown precipitate

III. SPECIMEN COLLECTION AND HANDLING:

A. Fixation
   1. Unfixed tissue that has been frozen.

B. Processing
   1. Fresh tissue.
   2. No processing.

C. Section Thickness
   1. Cut frozen sections at 10µ.

D. Storage
   1. Store slides in refrigerator.

E. Type of slide
   1. Plus slides.

IV. REAGENTS:

A. **0.2 M Sodium Succinate**

Sodium Succinate	**8.1 gm**
Distilled water	**250.0 ml**

Mix together. Store in brown bottle in refrigerator; expires in 1 year.

B. **0.2 Phosphate Buffer**

Sodium phosphate Dibasic, Anhydrous	**11.36 gm**
Potassium Phosphate Monobasic	**2.7 gm**
Distilled water	**500.0 ml**

Mix together well. Store in brown bottle in refrigerator; expires in 1 year.

C. **SDH Incubating Solution**

0.2 M Sodium Succinate	**10.0 ml**

0.2 M Phosphate buffer 10.0 ml
Nitro Blue Tetrazolium (NBT) 0.02gm

JUST BEFORE USE, mix together. Adjust pH to 7.2 – 7.6.

D. **1M Sodium Acetate Buffer, pH 5.6**
Sodium acetate, trihydrate 6.804 gm
Distilled water 500.0 ml
Dissolve together. Adjust pH to 5.6 with acetic acid. Store in refrigerator (4°C.); stable for months.

E. **1% Manganese Chloride**
Manganese chloride 1.0 gm
Distilled water 100.0 ml
Dissolve together. Store at room temperature; stable for months.

F. **0.1% Hydrogen Peroxide**
3% hydrogen peroxide 0.5 ml
Distilled water 14.5 ml
Mix together JUST BEFORE USE.

G. **1% Cupric Sulfate**
Cupric sulfate 1.0 gm
Distilled water 100.0 ml
Mix together. Store at room temperature; stable for months.

H. **1N Sodium Hydroxide**
Sodium hydroxide 4.0 gm
Distilled water 100.0 ml
Slowly add sodium hydroxide to water. Mix together; store at room temperature; stable for months.

I. **Incubating Medium**
DAB (3,3'-diaminobenzidine tetrahydrochloride 0.03 gm
1M sodium acetate buffer 13.5 ml
1% manganese chloride 1.5 ml
0.1% hydrogen peroxide 0.15 ml
Mix together JUST BEFORE USE. Adjust pH to 5.5 with 1N sodium hydroxide or concentrated acetic acid. Filter before use.

V. EQUIPMENT:

A. Metler balance
B. 60°C oven

VI. SUPPLIES:

A. Erlenmeyer flasks
B. Graduated cylinders
C. Funnel
D. Coplin jars

E. Beakers

F. Pipets

VII. SPECIAL SAFETY PRECAUTIONS:

A. Sodium Succinate (Succinic acid)

1. Is an irritant.

B. Nitro Blue Tetrazolium

1. Is an irritant.

C. Sodium Acetate

1. Is an irritant.

D. Acetic Acid

1. Is an acid.
2. Add slowly, drop by drop, to solution.
3. May cause skin and eye burns.

E. Manganese Chloride

1. Is an irritant
2. Is toxic.

F. Hydrogen Peroxide

1. Is an oxidizer.
2. Contact with other material may cause fire.
3. Store in refrigerator.
4. Can cause severe eye burns and skin burns.
5. Harmful if inhaled or swallowed.
6. Vapor is irritating to eyes and respiratory system.

G. Cupric Sulfate

1. Is an irritant.

H. DAB (3,3'-diaminobenzidine tetrahydrochloride)

1. Is a suspected carcinogen.
2. Wear gloves when preparing and using the incubating medium.

I. Sodium Hydroxide

1. Is corrosive and may cause severe eye and skin burns.

VIII. QUALITY CONTROL(QC):

Frozen section of muscle. (Built-in control, as all tissue has mitochondria).

IX. PROCEDURE:

Step	Action	Time	Notes
1	Pour SDH incubate solution over slides in plastic mailer.	2 hours (or longer)	Make solution just before use. Warm buffer to room temperature before use. Cover to prevent evaporation. Incubate in 37 °C oven. Tissues will appear blue after incubation.
2	Rinse in distilled water.	1 minute	
3	Place slides in cytochrome oxidase incubating medium solution.	1 hour	Make Just Before Use. Cover and incubate in a 37°C. oven. Incubating solution will be light brown in color. Tissue will appear light brown after incubation.
4	Rinse in distilled water, 2-3 changes.	5-10 seconds	
5	Place in 1% cupric sulfate.	5 minutes	
6	Rinse in distilled water, 2-3 changes.	30 seconds total	
7	Dehydrate through graded alcohols, clear in xylene.		
8	Coverslip using a synthetic mounting media.		

X. RESULTS:

A. Mitochondria - **brown**

B. Type I fibers - **darker brow**n

C. Type II fibers - **lighter brown**

D. Myofibrils - **unstained**

E. Mitochondria in cytochrome oxidase negative fibers - **blue**

XI. REFERENCES:

A. California Society of Histotechnology, May 16, 1987. Diagnostic Muscle Biopsy Procedure, Aldana Martin

B. Bancroft JD and Steven A: Theory and Practice of Histological Techniques, 3rd edition, New

York, NY. Churchill-Livingstone, 1990.

Approval Signatures

Step Description	Approver	Date
Medical Director	Kurt Bernacki: System Med Dir, Surgical Path	1/18/2023
Policy and Forms Steering Committee (if needed)	Sharon Scalise: Supv, Laboratory	1/17/2023
Policy and Forms Steering Committee (if needed)	Gail Juleff: Project Mgr Policy	1/17/2023
	Amy Knaus: Dir, Lab Operations C	1/17/2023
	Jennifer Lehmann: Mgr Laboratory	1/17/2023
	Sharon Scalise: Supv, Laboratory	1/10/2023

Applicability

Royal Oak